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Identification of a novel regulatory domain in Bcl-x_L and Bcl-2

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Bcl-x_L, a member of the Bcl-2 family, can inhibit many forms of programed cell death. The three-dimensional structure of Bcl-x_L identified a 60 amino acid loop lacking defined structure. Although amino acid sequence within this region is not conserved among Bcl-2 family members, structural modeling suggested that Bcl-2 also contains a large unstructured region. Compared with the full-length protein, loop deletion mutants of Bcl-x_L and Bcl-2 displayed an enhanced ability to inhibit apoptosis. Despite enhanced function, the deletion mutants did not have significant alterations in the ability to bind pro-apoptotic proteins such as Bax. The loop deletion mutant of Bcl-2 also displayed a qualitative difference in its ability to inhibit apoptosis. Full-length Bcl-2 was unable to prevent anti-IgM-induced cell death of the immature B cell line WEHI-231. In contrast, the Bcl-2 deletion mutant protected WEHI-231 cells from death. Substantial differences were observed in the ability of WEHI-231 cells to phosphorylate the deletion mutant of Bcl-2 compared with full-length Bcl-2. Bcl-2 phosphorylation was found to be dependent on the presence of an intact loop domain. These results suggest that the loop domain in Bcl-x_L and Bcl-2 can suppress the anti-apoptotic function of these genes and may be a target for regulatory post-translational modifications.

Keywords: apoptosis/bcl-x/bcl-2/programed cell death/ protein structure

Introduction

Apoptosis, or programed cell death (PCD), is an active process in which an individual cell responding to internal and/or external cues commits suicide (White, 1996; Yang and Korsmeyer, 1996). Cell death by apoptosis has several characteristic features, including nucleosomal DNA degradation, membrane blebbing and cell volume loss. PCD is involved in many diverse homeostatic processes in multicellular organisms, both during development and in the mature organism. Dysregulation of apoptosis can lead to pathological states involving cell accumulation, such as cancer, or cell loss, such as neurodegeneration (Thompson, 1995).

An increasing number of genes have been discovered that are involved in regulating apoptosis. A number of these genes belong to the *bcl-2* family. The original family member, *bcl-2*, was cloned from a lymphoma which overexpressed *bcl-2* as a result of a chromosomal translocation. Subsequent studies have demonstrated that Bcl-2 inhibits apoptosis in response to a variety of different stimuli, including irradiation, growth factor withdrawal, glucocorticoids, chemotherapeutic agents and viral infection. *bcl-2* appears to be the homologue of the *Caenorhabditis elegans* gene *ced-9*, which also has been shown to inhibit PCD (Hentgartner and Horvitz, 1994). In vertebrates, a number of *bcl-2*-related genes have been identified. One of these genes, *bcl-x*, has the ability to encode two distinct proteins as a result of alternative splicing, a long form (Bcl-x_L) and a short form (Bcl-x_S) (Boise *et al*., 1993). Bcl-x_L, like Bcl-2, inhibits apoptosis. In contrast, Bcl-x_S, which splices out 63 amino acids of highest homology to Bcl-2, can antagonize the anti-apoptotic action of Bcl-x_L and Bcl-2 (Minn *et al*., 1996). Several additional Bcl-2-related proteins, including Bax, Bad and Bak, have also been shown to potentiate apoptosis (Oltvai *et al*., 1993; Chittenden *et al*., 1995b; Farrow *et al*., 1995; Kiefer *et al*., 1995; Yang *et al*., 1995). Thus, the Bcl-2 family encompasses a diverse set of proteins spanning a large evolutionary distance and has members which can either inhibit or promote apoptosis.

One characteristic of this family of proteins is their propensity to form homo- and heterodimers (Oltvai *et al*., 1993; Sato *et al*., 1994; Hanada *et al*., 1995; Sedlak *et al*., 1995; Yang *et al*., 1995). Most family members can dimerize with themselves and/or with other family members. It is thought that the relative ratio of anti-apoptotic dimers versus pro-apoptotic dimers is important in determining the resistance of a cell to apoptosis. In addition to the associations between various family members, several other less well-characterized associations have been described between Bcl-2 family members and other proteins (Fernandez-Sarabia and Bischoff, 1993; Wang *et al*., 1994; Boyd *et al*., 1994; Chen and Faller, 1996).

Although several different theories have been proposed, the exact biochemical functions performed by the Bcl-2 family of proteins remain unclear. A diverse array of biochemical changes have been associated with apoptosis. Many of these changes, including the generation of reactive oxygen intermediates (Hockenbery *et al*., 1993; Kane *et al*., 1993), protease activation (Miura *et al*., 1993; Chinnaiyan *et al*., 1996; Fraser and Evan, 1996), calcium flux (Baffy *et al*., 1993; Lam *et al*., 1994) and mitochondrial membrane depolarization (Zamzami *et al*., 1995a,b, 1996), have been proposed to be activating signals for PCD. Bcl-2 has been shown to alter each of these

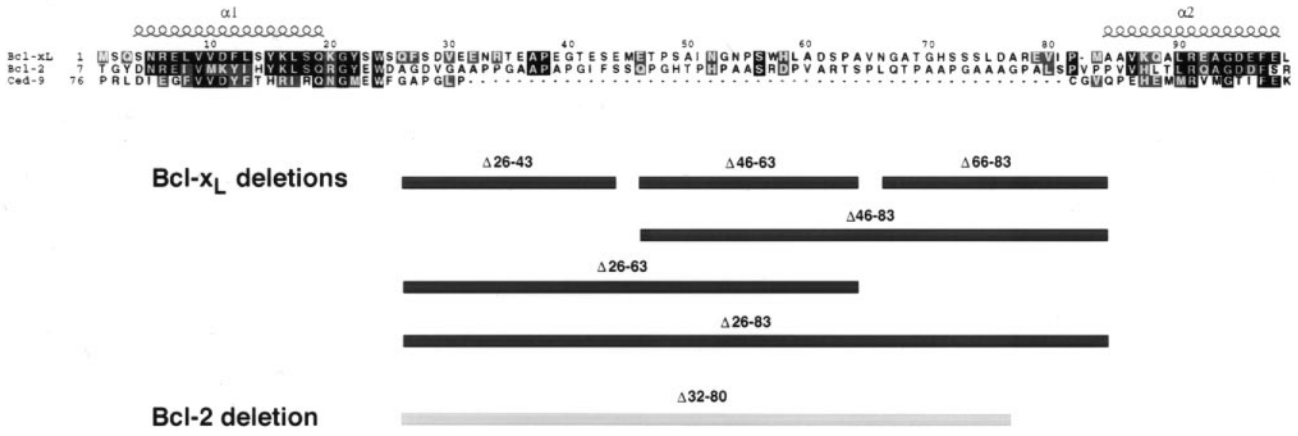

Fig. 1. Amino acid sequence comparison of the N-termini of human Bcl-x_L, human Bcl-2 and Ced-9. Numbers on top correspond to amino acid residues in Bcl-x_L. Alignments of Bcl-2 and Bcl-x_L with Ced-9 are based on previously described similarities (Hentgartner and Horvitz, 1994). The position of residues that comprise the α1 and α2 helices in the recently described three-dimensional structure of Bcl-x_L (Muchmore *et al*., 1996) are indicated above the sequence alignment. Dark gray boxes represent residues identical to Bcl-x_L, light gray represents amino acid similarity. Below, bars correspond to regions deleted from Bcl-x_L and Bcl-2 in the various deletion mutants used for subsequent analysis. All deleted residues were replaced with a linker comprised of four alanines.

biochemical changes. However, it has been difficult to determine whether this effect is a direct result of the biochemical action of Bcl-2 or simply a secondary result of inhibiting apoptosis.

Although Bcl-2 and Bcl-x_L are able to interfere with many forms of apoptosis, there are some forms of apoptosis that do not appear to be blocked by simple overexpression of Bcl-2. These include negative selection during lymphocyte development (Sentman *et al*., 1991; Strasser *et al*., 1991) and CNTF withdrawal from neurons (Allsopp *et al*., 1993). It is not clear why Bcl-2 is unable to block these death signals. One explanation may be that the induction of apoptosis following these death signals is fundamentally different from other forms of apoptosis inhibitable by Bcl-2 and Bcl-x_L. Alternatively, the biochemical function(s) of Bcl-2 and Bcl-x_L may be inactivated under such conditions.

The three-dimensional structure of Bcl-x_L has recently been obtained by X-ray crystallography and NMR spectroscopy (Muchmore *et al*., 1996). The structure consists of two central hydrophobic α-helices which are surrounded by amphipathic helices. In addition, Bcl-x_L contains a large unstructured loop of ~60 amino acids which is missing in the electron density map of the protein and which was shown to be highly flexible based on ^{15}N-^{1}H heteronuclear NOE experiments. Such a large unstructured region in a protein is unusual and prompted us to perform additional studies in order to determine how this domain might contribute to the biological properties of Bcl-x_L. To investigate whether this domain is important for the biological activities displayed by Bcl-x_L (and Bcl-2), several deletion mutants of Bcl-x_L and a deletion mutant of the comparable region in Bcl-2 were created. These mutants were examined for their ability to regulate apoptosis as well as their ability to form dimers with other Bcl-2 family members. These mutants all displayed enhanced anti-apoptotic properties that could not be explained by an increased ability to bind Bax. In addition to showing enhanced function, deletion of the loop region of Bcl-2 resulted in a protein that could inhibit a form of PCD previously reported to be unaffected by Bcl-2 overexpression. We propose that the loop region is a negative regulatory domain and may serve as a target for post-translational modifications that can selectively inhibit the biochemical function of Bcl-2 or Bcl-x_L.

Results

Deletion of the loop from Bcl-x_L leads to hyperfunction

During the course of determining the three-dimensional structure of Bcl-x_L, a large region of Bcl-x_L was found to be unstructured, as determined by both NMR and X-ray crystallographic techniques. This unstructured region is anchored on the N-terminus by the α1 helix and at the C-terminus by the α2 helix, both of which are key components of the compact α-helical bundle that comprises the rest of the molecule. This area of the molecule has been designated the loop domain. Amino acid sequence comparisons between Bcl-x_L, Bcl-2 and Ced-9 demonstrate that the amino acid sequence in this region is not conserved between Bcl-x_L and Bcl-2 and appears to be absent from Ced-9 (Figure 1).

To investigate the role of the loop domain in the anti-apoptotic function of Bcl-x_L, numerous overlapping and nested deletions were made within this domain of human Bcl-x_L (Figure 1). These deleted residues were uniformly replaced by a sequence of four alanines in place of the deleted residues to serve as a linker. The N-terminus of each protein was tagged with a hemagglutinin (HA) epitope. An N-terminal tagged version of full-length Bcl-x_L was also produced for comparison. No difference in the anti-apoptotic function of Bcl-x_L with or without the HA tag was observed (data not shown). Evidence from several groups also suggests that N-terminal epitope tags of Bcl-2 family members have no effect on protein function (Chittenden *et al*., 1995b; Yang *et al*., 1995; Minn *et al*., 1996).

The anti-apoptotic function of six Bcl-x_L deletion constructs was tested following stable transfection into murine FL5.12 cells. These pro-B cells are IL-3 dependent and undergo apoptotic cell death upon IL-3 deprivation (Nuñez *et al*., 1990). As Figure 2A shows, the control transfectants

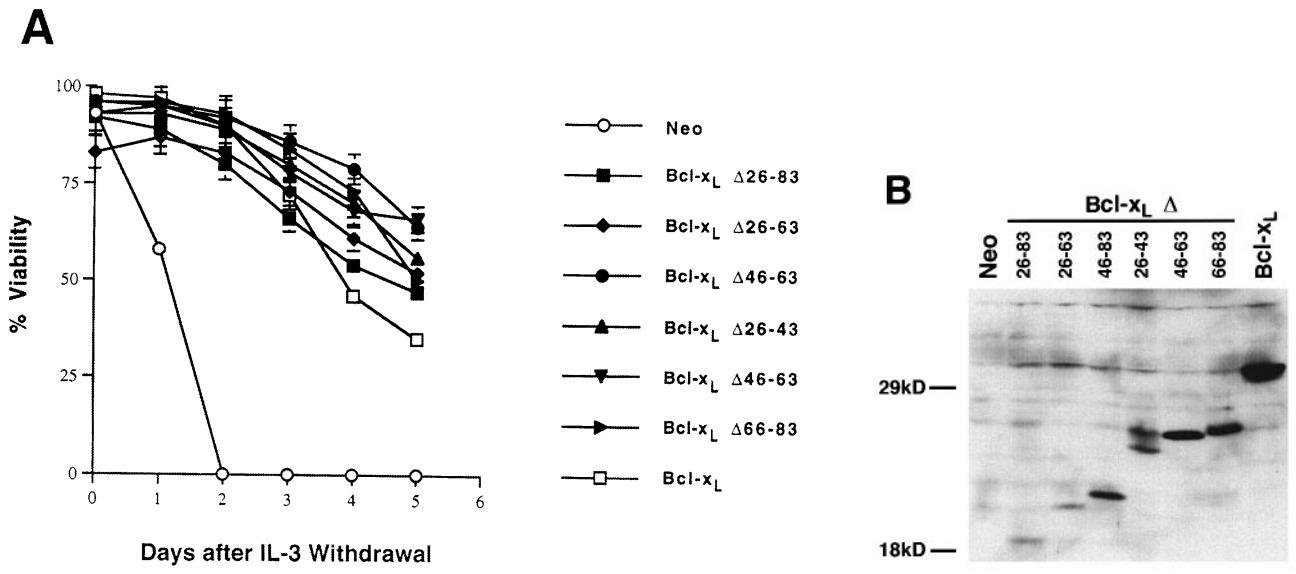

Fig. 2. Deletions of the loop domain of Bcl-x_L do not abrogate protective function. Murine FL5.12 cells were transfected with Bcl-x_L constructs containing the indicated deletions. (**A**) Percent viability over time of the bulk transfectants was examined after IL-3 deprivation. Viability was determined by propidium iodide exclusion as measured by flow cytometry. Survival of cells transfected with the control vector (Neo) is also indicated. Means of three independent determinations are shown. Error bars represent one standard deviation. These results are representative of three independent experiments. (**B**) Expression of Bcl-x_L deletion proteins in FL5.12 cells. All Bcl-x_L constructs were N-terminally tagged with a HA epitope. Bulk transfectants of the Bcl-x_L constructs containing the indicated deletions were examined for Bcl-x_L expression by Western blot analysis using a mouse monoclonal antibody (12CA5) specific for the N-terminal HA tag.

undergo apoptosis rapidly upon IL-3 deprivation, while the full-length Bcl-x_L-transfected cells are significantly protected. Unexpectedly, bulk transfectants of all six deletion constructs showed reproducible enhancement of anti-apoptotic function relative to the full-length Bcl-x_L protein. This enhancement of function could not be explained simply by the relative levels of protein expression as assayed by Western blot (Figure 2B). Each of the deletion proteins was found to be expressed at levels equal to or lower than the full-length Bcl-x_L protein. In addition, unlike full-length Bcl-x_L, which migrates as a discrete doublet, all the deletion mutants except Δ26–43 migrated on SDS–PAGE gels as a sharp, single band. The molecular basis for this difference in migration pattern has not been determined.

To confirm that the transfectants of the Bcl-x_L deletion constructs demonstrate enhanced anti-apoptotic function, clones of these transfectants were generated by limiting dilution. Clones of two of the largest deletion mutants, Bcl-x_L Δ46–83 and Bcl-x_L Δ26–83, were then studied in greater detail. Clones were also generated from the full-length Bcl-x_L transfectants as positive controls. Protein expression of the three sets of clones generated is shown in Figure 3D. Similar to the bulk transfectants, the deletion mutants were both underexpressed relative to the full-length Bcl-x_L protein and migrated as single bands. Even though all constructs were under the control of the same spleen focus forming virus (SFFV) promoter, we were unable to generate clones of the deletion mutants with levels of expression equal to clones of the full-length protein, as quantitated by Western blot using either anti-HA or anti-Bcl-x_L monoclonal antibodies. Therefore, clones of Bcl-x_L with relatively low levels of expression were chosen for comparison. Nevertheless, despite the low expression, both deletion mutants confer a level of resistance to apoptosis that was significantly greater than the resistance imparted by full-length Bcl-x_L (Figure 3). The two full-length Bcl-x_L clones had a viability of $<$15% at 7 days after factor withdrawal, while the Δ26–83 clones were $>$55% viable and the Δ46–83 clones were $>$67% viable at this time point. Several additional clones of the three constructs were also tested and were found to protect against apoptosis similarly to the clones presented (data not shown). The failure of full-length Bcl-x_L to protect as completely from apoptosis as the deleted clones was not the result of expression of Bcl-x_L above an optimum level for cell protection. Bcl-x_L clones that expressed 2- to 3-fold more full-length Bcl-x_L than clones shown in Figure 3A displayed a significantly greater ability to support cell survival 7 days after factor withdrawal (Figure 3E). Clones that displayed lower levels of full-length Bcl-x_L have levels of survival that approach those of neomycin control transfectants (data not shown; Minn *et al*., 1996). These data suggest that the unstructured region of Bcl-x_L exerts a negative effect on the protective function of Bcl-x_L. Deletion of this region removes this negative regulation, resulting in enhanced anti-apoptotic function.

Bax heterodimerization is unaffected by loop deletion

Although the biochemical mechanism of Bcl-x_L action is unknown, one proposed model suggests that heterodimerization of Bcl-x_L with other Bcl-2 family members may be important in regulating the apoptotic threshold of a cell (Oltvai and Korsmeyer, 1994; Yin *et al*., 1994; Yang *et al*., 1995). In this model, the heterodimerization of Bcl-x_L with the pro-apoptotic family members such as Bax, Bak and Bad is essential for its anti-apoptotic effect. One possible explanation for the hyperfunction of the deletion mutants is that these proteins are better able to heterodimerize with the death-promoting family members and thereby block their ability to potentiate apoptosis. To

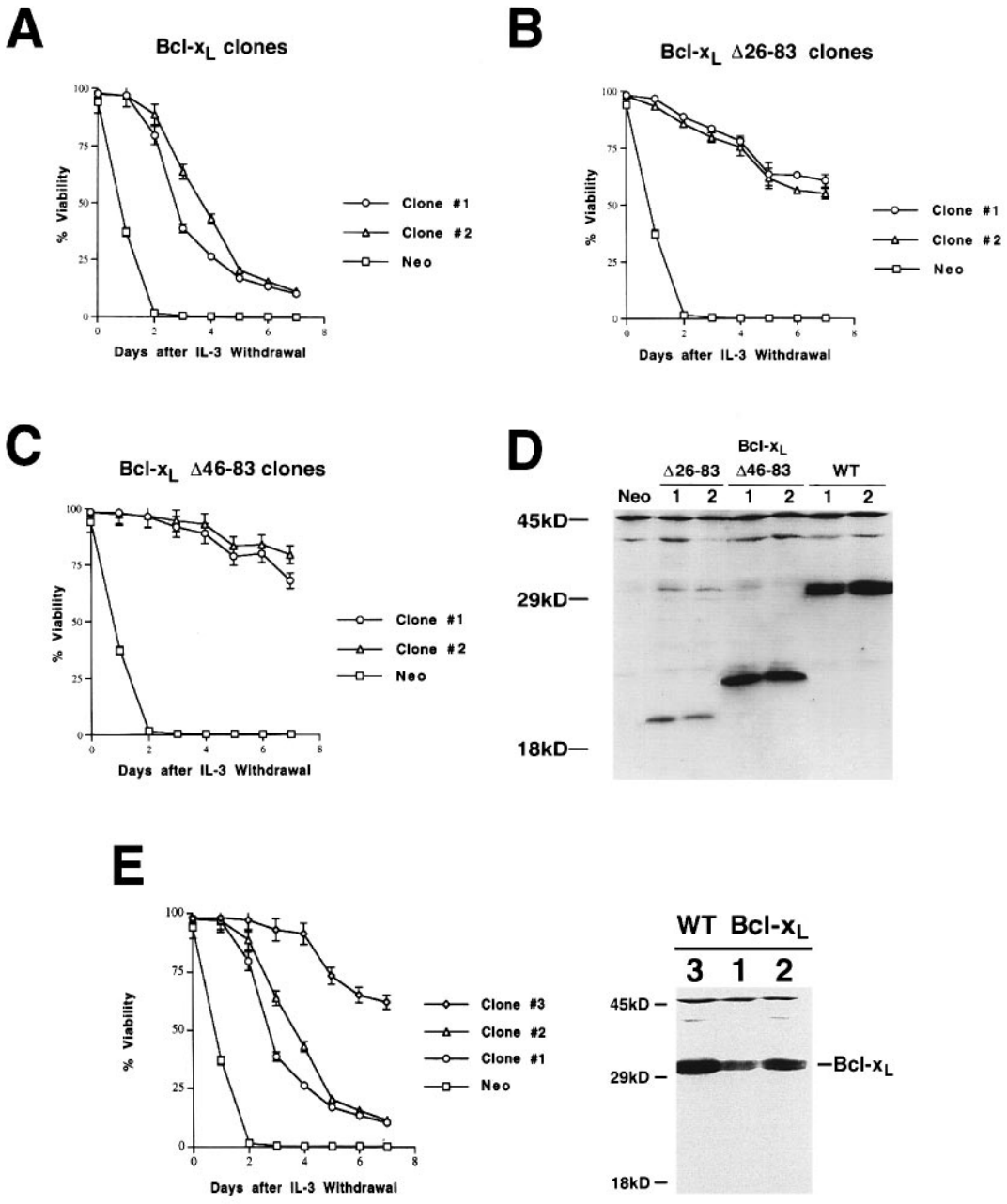

Fig. 3. Comparison of viability between clones of FL5.12 expressing either Bcl-x_L, Bcl-x_L Δ26–83 or Bcl-x_L Δ46–83 following IL-3 withdrawal. Single cell clones of the full-length protein and two deletion proteins, Bcl-x_L Δ26–83 and Bcl-x_L Δ46–83, were generated by limiting dilution from the bulk transfectant population. Percent viability over time following IL-3 deprivation was measured by propidium iodide exclusion. Means of three independent determinations are shown. Error bars represent one standard deviation. These results are representative of two independent experiments. (**A**) Bcl-x_L. (**B**) Bcl-x_L Δ26–83. (**C**) Bcl-x_L Δ46–83. The survival of control transfectants (Neo) in the same experiment is also indicated. (**D**) Expression levels of full-length Bcl-x_L and deletion proteins in the various clones was determined by Western blot using a monoclonal antibody specific for the N-terminal HA tag expressed by each construct. WT indicates cells transfected with a full-length Bcl-x_L construct. (**E**) Enhanced survival of the Bcl-x_L deletion clones can be mimicked by higher levels of full-length Bcl-x_L. The survival of Bcl-x_L clone 3, which expresses 2- to 3-fold higher levels of Bcl-x_L than Bcl-x_L clones 1 and 2, displays survival properties similar to those observed for the Bcl-x_L Δ26–83 clones. The level of Bcl-x_L in Bcl-x_L clone 3 in comparison with Bcl-x_L clones 1 and 2 as determined by Western blotting is presented on the right.

test this possibility, all six loop mutant transfectants, as well as full-length Bcl-x_L-transfected FL5.12 cells, were metabolically labeled with [^{35}S]methionine. Cell lysates were immunoprecipitated with an anti-HA antibody (12CA5) and the amount of associated protein was determined. As reported previously, the major Bcl-2 homolog associated with Bcl-x_L in FL5.12 cells was Bax (Minn *et al*., 1996). As shown in Figure 4A, all of the deletion mutants retain the ability to immunoprecipitate Bax. The ratio of Bax to Bcl-x_L in the immunoprecipitates is relatively constant, suggesting that the ability to dimerize with Bax is not substantially affected in loop deletion mutants. However, it should be noted that due to the lower levels of Bcl-x_L mutant proteins expressed in the transfectants, the absolute quantity of Bax heterodimerized with the Bcl-x_L deletion mutants is less than the quantity of Bax associated with full-length Bcl-x_L. For example, as measured by scanning densitometry, full-length Bcl-x_L

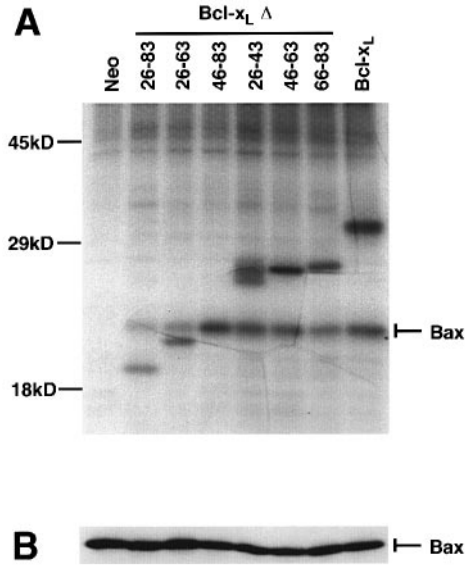

Fig. 4. Association of Bax with Bcl-x_L deletion mutants. (**A**) FL5.12 cells transfected with the indicated Bcl-x_L constructs were metabolically labeled overnight with [^{35}S]methionine. Cell lysates were then immunoprecipitated with an anti-HA antibody. Immunoprecipitates were separated by SDS–PAGE and exposed by fluorography. The position of Bax is indicated to the right of the gel. Bcl-x_L Δ46–83 migrates at the same position as Bax. Neomycin (Neo)-resistant control transfectants were subjected to similar immunoprecipitation to confirm the specificity of the Bax association. The ratio of Bax to Bcl-x_L in the immunoprecipitates was measured by scanning densitometry and corrected for the methionine content of the Bcl-x_L deletion mutants. The ratio of Bax to Bcl-x_L was 0.59 for full-length Bcl-x_L, 0.57 for Δ26–83, 0.61 for Δ26–63, 0.42 for Δ26–43, 0.60 for Δ46–63 and 0.63 for Δ66–83. The ratio of Bax to Bcl-x_L Δ46–83 could not be estimated due to co-migration of the two proteins. (**B**) Cell lysates from each of the Bcl-x_L transfectants were analyzed by Western blot using an anti-Bax rabbit polyclonal antibody N-20 (Santa Cruz) to determine endogenous Bax expression levels. Lanes were loaded as indicated in (A).

immunoprecipitated 2.6-fold more Bax than did Bcl-x_L Δ26–83. None of the mutants immunoprecipitated more Bax than did the full-length protein. Since the level of Bax expressed in the cells is equivalent (Figure 4B), the absolute amount of Bax unassociated with Bcl-x_L, (i.e. 'free' Bax) is greater in the cells expressing the deleted proteins as compared with the cells expressing full-length Bcl-x_L.

The heterodimerization capabilities of Bcl-x_L and a Bcl-x_L loop deletion mutant were further investigated *in vitro*. The BH3 domain of Bak has been found to be necessary and sufficient to mediate dimerizations between Bcl-x_L and Bak (Chittenden *et al*., 1995a). Interactions between Bcl-x_L or Bcl-x_L Δ45–84 with a 23 amino acid peptide sequence (GQVGRQLAIIGDDINRRYDSEFQ) representing the BH3 region of Bak were measured by tryptophan fluorescence quenching. The binding constants (K_D) for Bcl-x_L and Bcl-x_L Δ45–84 were both determined to be 200 nM. Thus, deletion of the loop domain does not alter the ability of Bcl-x_L to associate with Bax or Bak.

Loop deletions in Bcl-2 also lead to increased function

Given the extensive sequence identity between Bcl-x_L and Bcl-2, it is expected that the three-dimensional structure of Bcl-2 will be very similar to the recently described structure of Bcl-x_L. Based on modeling, Bcl-2 likely contains a large unstructured loop region between helices α1 and α2. However, the amino acid residues within this predicted loop region are very different in the two proteins. To test if this region in Bcl-2 also imparts a negative effect on its anti-apoptotic function, a construct was made in which 49 amino acids (32–80) were deleted from this region of Bcl-2 and replaced with a linker of four alanines. An HA epitope tag was also added at the N-terminus. FL5.12 cells were transfected with the Bcl-2 deletion (Bcl-2Δ) construct, a full-length Bcl-2 construct and a control plasmid containing only a neomycin resistance gene. From these transfected cells, clones were generated expressing either the Bcl-2Δ or the full-length Bcl-2 construct. These clones were screened for Bcl-2 using a polyclonal anti-Bcl-2 antibody recognizing a peptide sequence shared by both constructs (Figure 5B). As with the Bcl-x_L deletions, clones with equivalent expression of the deletion and full-length Bcl-2 protein could not be generated. However, despite the much higher expression of the full-length Bcl-2 protein, when these clones were tested by IL-3 withdrawal (Figure 5A), the deletion protein conferred a greater protective effect against apoptosis than the full-length protein. Thus, the negative regulatory function of the putative unstructured loop region seems to be a shared characteristic of Bcl-x_L and Bcl-2.

To test the ability of the full-length and the mutant Bcl-2 to heterodimerize with endogenous Bax, the FL5.12 transfectants were metabolically labeled with [^{35}S] methionine. To assure qualitatively as well as quantitatively similar immunoprecipitations, both proteins were immunoprecipitated using an anti-HA antibody. Figure 6 shows the results of the immunoprecipitation. No significant difference in the amount of Bax that could be co-immunoprecipitated was observed. Similar results were obtained using non-epitope-tagged Bcl-2 (data not shown).

Deletion of the putative loop in Bcl-2 restores function in the WEHI-231 cell line

The murine immature B cell line WEHI-231 has previously been shown to undergo apoptosis upon IgM crosslinking and this system has been used as a model of negative selection (DeFranco *et al*., 1987; Hasbold and Klaus, 1990; Cuende *et al*., 1993). Apoptosis in these cells is inhibitable by Bcl-x_L but not by Bcl-2 (Gottschalk *et al*., 1994; Choi *et al*., 1995). Since the negative regulatory loops in Bcl-2 and Bcl-x_L bear no significant similarity to each other, one possibility is that differences in the loop domains might account for the observed functional difference between Bcl-2 and Bcl-x_L in WEHI-231 cells. To test whether the qualitative difference in function of Bcl-2 and Bcl-x_L in WEHI-231 cells resulted from the differences in the loop domains, the deletion construct of Bcl-2 was tested in this system. WEHI-231 cells were stably transfected with Bcl-2Δ and Bcl-2 and clones were derived by limiting dilution. Clones with roughly equivalent expression levels of Bcl-2 and Bcl-2Δ were isolated (Figure 7C) and tested for their resistance to anti-IgM-induced death. Figure 7A demonstrates the failure of full-length Bcl-2 to inhibit apoptosis due to IgM crosslinking. Analysis of multiple clones from three separate transfections demonstrated that Bcl-2 transfectants reproducibly survive less well than control transfectants

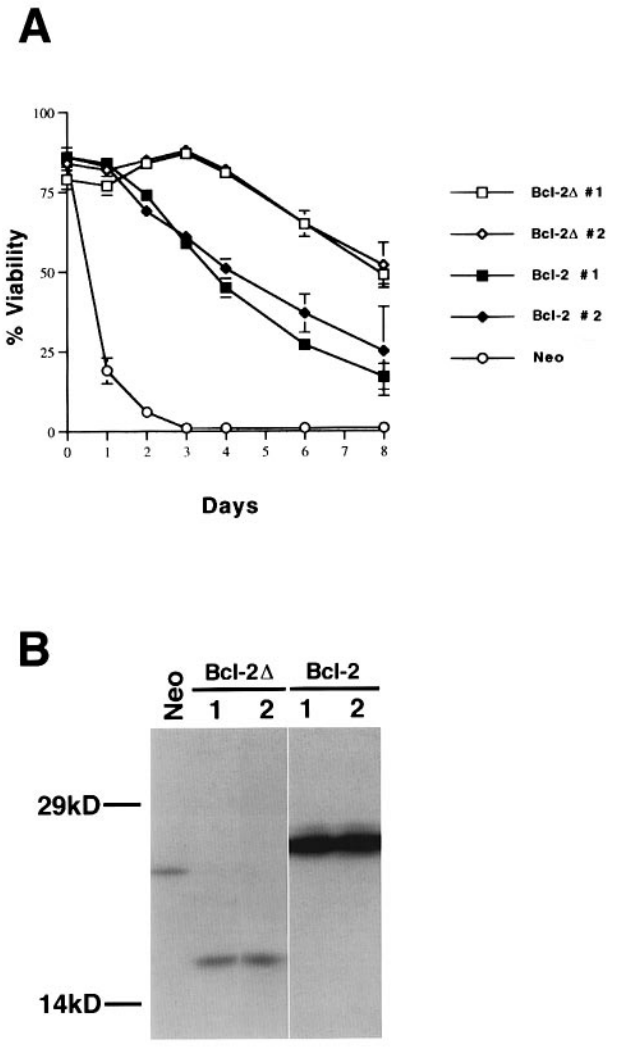

Fig. 5. Viability and expression of Bcl-2Δ and Bcl-2 in FL5.12 cells. Cells were transfected with either Bcl-2Δ or Bcl-2 expression constructs and clones were derived by limiting dilution. (**A**) Viability assays following IL-3 withdrawal were performed as described in the legend to Figure 2. Means of three independent determinations are shown. Error bars represent one standard deviation. Results are representative of two independent experiments. Neo indicates the survival of cells transfected with a control construct encoding a neomycin resistance gene. (**B**) Expression levels of Bcl-2 and Bcl-2Δ in the clones was determined using a polyclonal rabbit antibody which recognizes both murine and human Bcl-2 equivalently. The band observed in the cells transfected with a neomycin-containing control plasmid (Neo) is murine Bcl-2. As previously reported, its endogenous expression is suppressed by expression of transfected Bcl-2 (Chao *et al*., 1995).

even at the highest levels of expression obtainable. In contrast, Bcl-x_L was able to impart resistance to cell death in response to IgM crosslinking. Deletion of the putative loop domain from Bcl-2 converts it from a non-protective protein into a protein which prevented anti-IgM-induced apoptosis at least as well as Bcl-x_L (Figure 7B). These data suggest that the putative loop domain of Bcl-2 completely abrogates the protective function of Bcl-2 in WEHI-231 cells. In contrast, the loop domain of Bcl-x_L does not abrogate Bcl-x_L function in this cell line. Consistent with these data, transfection of a chimeric construct, engineered by replacing the N-terminal half of Bcl-2, which includes the putative loop domain, with the comparable region of Bcl-x_L, was also able to inhibit anti-IgM-induced apoptosis in WEHI-231 cells. Transfection of the reciprocal construct containing the N-terminus of Bcl-2 fused to the C-terminus of Bcl-x_L failed to inhibit anti-IgM-induced apoptosis (data not shown).

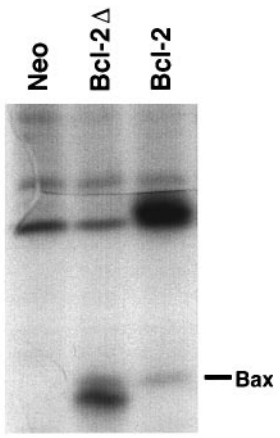

Fig. 6. Association of Bax with Bcl-2 and Bcl-2Δ in FL5.12 cells. Cells were metabolically labeled with [^{35}S]methionine overnight and cell lysates were immunoprecipitated with an anti-HA antibody specific for the N-terminal HA tag present in both Bcl-2 and Bcl-2Δ. Immunoprecipitates were resolved by SDS–PAGE. The position of co-immunoprecipitated Bax is indicated. Control transfectants expressing a neomycin resistance gene (Neo) were also subjected to immunoprecipitation with the anti-HA antibody. Bands in this lane represent proteins non-specifically immunoprecipitated.

The ability of Bcl-2 and Bcl-2Δ to form heterodimers in WEHI-231 cells was also examined by immunoprecipitation. As in FL5.12 cells, no difference in Bax heterodimerization ability was found between Bcl-2 and Bcl-2Δ (data not shown).

Deletion of the loop domain alters the ability of Bcl-2 to be phosphorylated

Serine/threonine phosphorylation of Bcl-2 has been reported to be associated with a decrease in biological function (Haldar *et al*., 1995). We therefore sought to investigate whether phosphorylation in the loop is responsible for the negative modulation of Bcl-x_L and/or Bcl-2 function. In order to observe ongoing serine/threonine phosphorylation, Bcl-2- and Bcl-2Δ-transfected FL5.12 and WEHI-231 cells were labeled with [^{32}P]orthophosphate in the presence of okadaic acid. Immunoprecipitation revealed phosphorylation of Bcl-2 but not Bcl-2Δ in both FL5.12 and WEHI-231 cells (Figure 8). Interestingly, the phosphorylation pattern as determined by mobility shift on a SDS–PAGE gel is different between the two cell lines. Phosphorylation as judged by both gel mobility shifts and band intensity was much more extensive in WEHI-231 cells, suggesting that either a different degree of phosphorylation or differential sites of phosphorylation occurred in these two cells lines. Under similar conditions, no phosphorylation of Bcl-2Δ was detectable in either cell line and Bcl-2Δ ran as a sharp single band. We were unable to detect phosphorylation of Bcl-x_L under similar conditions (data not shown). Thus, the loop domain of Bcl-2 is either a substrate for phosphorylation or contains a binding site for a kinase(s) that acts elsewhere on the molecule. Deletion of the loop is sufficient to inhibit the phosphorylation of Bcl-2.

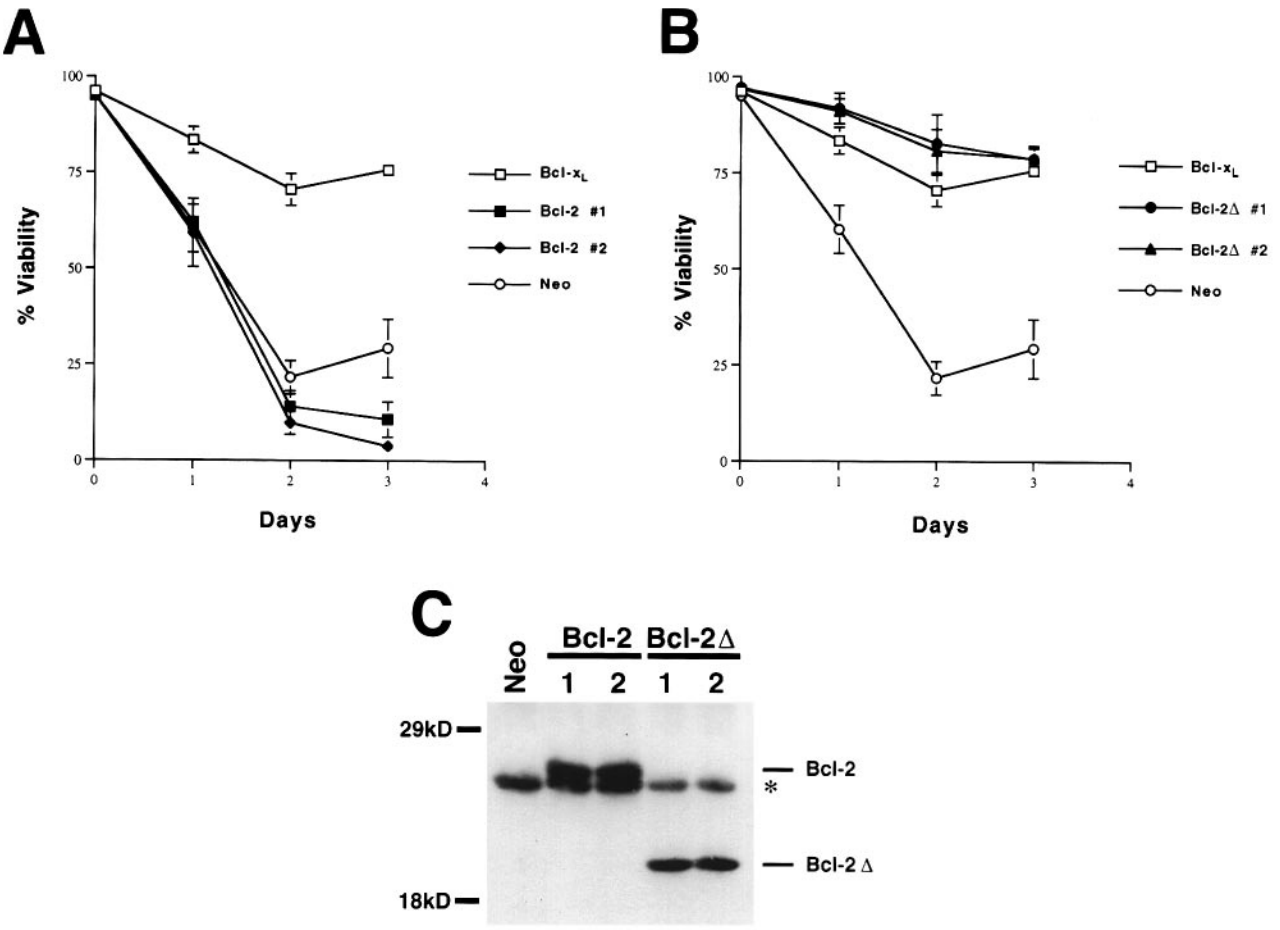

Fig. 7. Bcl-2Δ but not Bcl-2 protects WEHI-231 cells from anti-IgM-induced apoptosis. WEHI-231 cells were transfected with either a Bcl-2 or Bcl-2Δ expression construct and single cell clones were derived by limiting dilution. For viability assays cells were incubated in 24-well plates at 5×10^5 cells/ml and treated with the anti-IgM antibody BET2. Viability was measured by propidium iodide exclusion. Means of three independent experiments are shown. Error bars represent one standard deviation. (**A**) Bcl-2-transfected WEHI-231 cells do not exhibit enhanced protection relative to control transfectants containing a neomycin resistance gene (Neo). In contrast, Bcl-x_L transfectants displayed a high degree of protection against anti-IgM-induced apoptosis. (**B**) Bcl-2Δ transfectants show a high level of resistance to anti-IgM-induced apoptosis. (**C**) Expression of Bcl-2 in the indicated clones was determined by Western blot analysis with an anti-Bcl-2 antibody (N-19). The positions of transfected human Bcl-2 and Bcl-2Δ are indicated. An asterisk denotes endogenous murine Bcl-2.

Discussion

Previous mutagenesis and deletional analysis of Bcl-2 family members has concentrated primarily on the regions of homology: BH1, BH2 and BH3. These regions have been shown to be involved in mediating dimerizations between the various family members (Yin *et al*., 1994; Chittenden *et al*., 1995a; Zha *et al*., 1996; Sattler *et al*., 1997). However, the data presented here suggest that independent of heterodimerization, anti-apoptotic function can be regulated by the large loop domain of Bcl-x_L and its putative homologous domain in Bcl-2. Deletion of the loop domain in the two different genes leads to a quantitative increase in protection of FL5.12 cells against IL-3 withdrawal-induced death. Furthermore, the presence of the negative regulatory loop in Bcl-2 accounts for the failure of the full-length protein to protect against anti-IgM-induced death of WEHI-231 cells. This qualitative difference in Bcl-2 function in WEHI-231 cells correlates with the inability of WEHI-231 cells to phosphorylate the Bcl-2Δ protein.

The ability of the loop deletion mutants to enhance both the quantitative and qualitative function of Bcl-2 and Bcl-x_L cannot be accounted for by differences in the ability of the loop deletion proteins to bind Bax. The stoichiometry of binding to Bax in immunoprecipitates is not affected by deletion mutants in Bcl-x_L. Furthermore, the binding affinity of Bcl-x_L for the Bak BH3 peptide, which has been shown to inhibit Bcl-x_L function in cells, is not altered by deletion of the Bcl-x_L loop domain. In FL5.12 cells, the Bcl-x_L Δ26–83 mutant provides better protection from apoptosis than full-length Bcl-x_L, even though the deletion mutant is expressed at much lower levels. Since Bcl-x_L and Bcl-x_L Δ26–83 both bind Bax stoichiometrically and total Bax levels are identical between the two transfectants, the total amount of unheterodimerized or ‘free’ Bax must be greater in the deletion mutant transfectants than in the full-length Bcl-x_L transfectants. These data suggest that Bcl-x_L does not protect cells merely through its ability to bind Bax and thereby prevent the formation of Bax homodimers. These data are consistent with a previous finding that Bcl-x_L has anti-apoptotic function in the absence of Bax or Bak binding capability (Cheng *et al*., 1996).

The loop domain is not conserved between Bcl-x and

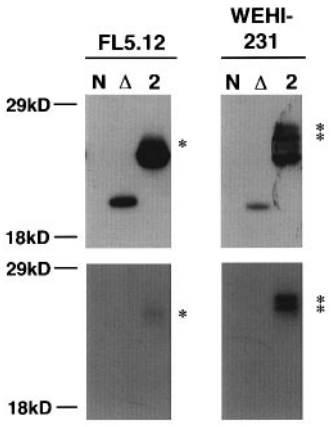

Fig. 8. Bcl-2 but not Bcl-2Δ is extensively phosphorylated in WEHI-231 cells. FL5.12 and WEHI-231 cells transfected with either an empty control vector (Neo), a Bcl-2-expressing construct or a Bcl-2Δ construct were metabolically labeled with 0.5 mCi [^{32}P]orthophosphate in the presence of 1 μM okadaic acid to inhibit serine/threonine phosphatase activity. Cells were lysed and immunoprecipitates of Bcl-2 (2) or Bcl-2Δ (Δ) were detected by Western blotting with anti-Bcl-2 antibody N-19 (top). After allowing the ECL signal to decay, the same blot was then exposed to autoradiography to detect incorporated ^{32}P (bottom). Asterisks denote ^{32}P-containing bands detected by both assays.

Bcl-2 and most of the intraspecies divergence of the two genes occurs in this region. Published alignments of another anti-apoptotic gene product, the *C.elegans* protein Ced-9, suggest that this protein does not have a sufficient number of amino acids to encode a regulatory loop region between its putative α1 and α2 helices (Hengartner and Horvitz, 1994). Unlike higher organisms, *C.elegans* appears to have only a single *bcl-2*-related gene. Thus, Ced-9 may not need a specialized domain to post-translationally control its function. However, higher organisms seem to have acquired a more intricate system for regulating Bcl-2 function. This may be the reason that higher organisms have evolved additional family members that regulate apoptosis via differential dimerization, as well as through gene-specific regulatory domains as defined here for Bcl-2 and Bcl-x_L.

Phosphorylation of proteins is a ubiquitous mechanism utilized by cells to regulate protein activity. Phosphorylation of the loop domain of Bcl-2 must exclusively involve serine/threonine, as there are no tyrosines in the loop domain (Figure 1). Serine phosphorylation of Bcl-2 has been reported to correlate with an increase in anti-apoptotic activity by two groups (May *et al*., 1994; Chen *et al*., 1995; Cheng *et al*., 1996) and with a decrease by another (Haldar *et al*., 1995). Although the data presented here do not resolve this debate, there does seem to be a correlation between phosphorylation of Bcl-2 and decreased anti-apoptotic activity. Furthermore, phosphorylation may not turn off Bcl-2 function in a single step. Although Bcl-2 is phosphorylated in both cell lines examined, albeit less intensely in FL5.12, Bcl-2 retains anti-apoptotic function only in FL5.12 cells, while it is non-functional in WEHI-231 cells. The failure of Bcl-2 to protect WEHI-231 cells is not due to a Bcl-2-insensitive apoptotic pathway, since deletion of the negative regulatory loop restores the ability of Bcl-2 to prevent apoptosis in WEHI-231 cells. Indeed, the mobility of phosphorylated Bcl-2 on SDS–PAGE shows a significant difference between the two cell lines examined in this study, arguing that there can be variations in the degree of phosphorylation. As there are five serines and three threonines in the loop domain of Bcl-2, it is possible that there are multiple phosphorylation sites present in the loop. Alternatively, it cannot be ruled out that the loop domain is not the substrate for phosphorylation, but instead is necessary for recognition by a kinase that then phosphorylates a different region of the protein.

Proteins containing regions of denatured or random coil structure do not normally exhibit long half-lives, due to recognition and cleavage by cellular proteases (Ciechanover, 1994). Therefore, it is likely that the large unstructured loop region is shielded or otherwise protected from rapid degradation by other associated proteins. These putative loop-associating proteins might either directly modulate the function of Bcl-2 and Bcl-x_L or, alternatively, regulate their accessibility to protease digestion. Consistent with this latter possibility, unpublished data from our and other laboratories suggest that the Bcl-x_L and Bcl-2 loop domains can be targets for proteases that are activated during apoptosis. Interestingly, three novel proteins have been cloned via a yeast two-hybrid screen whose interaction with Bcl-2 was reported to be dependent upon a short stretch of amino acids within the putative loop domain (Boyd *et al*., 1994).

Finally, the effect of the loop domain on Bcl-2 and Bcl-x_L function may be variable depending on the cell type and death signal. As this loop domain is very large, comprising one quarter of these proteins, there is ample room for multiple interaction sites which could lead to complex and independent regulation of Bcl-2 and Bcl-x_L function. The regulation of Bcl-x_L and Bcl-2 function by post-translational mechanisms would allow for dynamic alterations in the apoptotic threshold of a cell even under conditions of high Bcl-x_L or Bcl-2 expression. Constitutively activating Bcl-2 and Bcl-x_L, by deletion of the loop domain, may override the ability of certain cells to inactivate these proteins by post-translational mechanisms. Tumor cells would have a great selective advantage if they could create hyperfunctional mutants of Bcl-2 and Bcl-x_L which are not susceptible to inactivation. Several reports have documented mutations in the putative loop domain of Bcl-2 in DNA isolated from tumor specimens (Tanaka *et al*., 1992a,b). Thus, alterations in the negative regulatory properties of the loop domain may be another mechanism by which modifications in the function of Bcl-2 proteins may contribute to carcinogenesis.

Materials and methods

Plasmid constructs

Deletion mutants of Bcl-x_L and Bcl-2 were generated by two-step recombinant PCR (Erlich, 1989) and confirmed by sequencing. An influenza hemagglutinin epitope (MDYPYDVPDYA-) was added to the 5′-end of some constructs by PCR. These constructs were cloned into the plasmid pBluescript II SK+ (Stratagene), sequenced and then subcloned into the expression vector pSFFV-Neo (Boise *et al*., 1993).

Cell lines and transfection

The murine cell lines FL5.12 and WEHI-231 were cultured as described previously (Boise *et al*., 1993; Gottschalk *et al*., 1994). Transfections with various constructs in pSFFV-Neo were performed using 10 μg plasmid, electroporated into 1×10^7 cells at 960 μF and 250 V. Neomycin-

resistant cells were selected with 1 mg/ml G418. Single cell clones were isolated from the bulk transfectants by limiting dilution cloning in 96-well microtiter plates.

Western blot analysis of proteins

Cells were pelleted by centrifugation and washed with phosphate-buffered saline, then lysed with RIPA buffer (1% Nonidet P-40, 1% deoxycholate, 0.1% SDS) supplemented with 8 μg/ml aprotinin, 2 μg/ml leupeptin and 170 μg/ml phenylmethylsulfonyl flouride (PMSF). After cellular debris was removed by centrifugation at 14 000 *g*, the protein concentration of the supernatant was determined using colorimetric bicinchoninic acid analysis (BCA; Pierce). Equal quantities of proteins were denatured in SDS sample buffer and separated by 15% SDS–PAGE. After transfer to nitrocellulose, blots were blocked with 5% milk with either 0.1 or 0.05% Tween-20. Blots were then incubated with either 12CA5 anti-HA monoclonal antibody (4 μg/ml; Boehringer Mannheim) in 5% milk with 0.1% Tween-20, N-19 polyclonal anti-Bcl-2 antibody (0.5 μg/ml; Santa Cruz) in 5% milk with 0.05% Tween-20 or N-20 polyclonal anti-Bax antibody (0.5 μg/ml) (Santa Cruz) in 5% milk with 0.05% Tween-20. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies were incubated secondarily (Amersham). Western blots were developed using ECL (Amersham).

Cell viability assays

FL5.12 cell viability was assayed by IL-3 deprivation. Cells were washed three times with medium lacking WEHI 3B supernatant (a source of IL-3) and then resuspended in this medium at 5×10^5 cells/ml. Aliquots were taken over the course of the experiment and viability was measured by propidium iodide exclusion, as previously described (Boise *et al*., 1993). WEHI-231 cell viability was measured by propidium iodide exclusion in a similar fashion, following treatment with the anti-IgM antibody BET2 supernatant at a 1:50 dilution.

^{35}S metabolic labeling and immunoprecipitations

Cells were collected during log phase growth, washed with fresh medium and resuspended at 10^6 cells/ml. They were then labeled with 0.5 mCi Trans-label [^{35}S]methionine (ICN) overnight. Cells were lysed in 500 μl NET-N buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris, 0.2% NP-40, pH 8.0) supplemented with 8 μg/ml aprotinin, 2 μg/ml leupeptin and 170 μg/ml PMSF. Lysates were centrifuged at 14 000 *g* to remove debris. The supernatant was precleared with 25 μl protein G–agarose (Gibco BRL) for 1 h at 4°C on a rocking platform. Ten micrograms of 12CA5 anti-HA monoclonal antibody or 2 μg 6C8 anti-Bcl-2 monoclonal antibody (Pharmingen) were added to the supernatant and rocked for several hours at 4°C. Twenty five microliters of protein G–agarose was then added and rocked for another hour at 4°C. The agarose was centrifuged at 14 000 *g* and washed three times with NET-N buffer. The proteins were released and denatured by addition of SDS sample buffer and heating at 95°C for 5 min. Samples were subject to separation by 15% SDS–PAGE. Gels were treated with Amplify (Amersham), dried and exposed to film at –70°C.

In vitro fluorescence titrations

The peptide binding affinities of Bcl-x_L and Bcl-x_L Δ45–84 were measured by fluorescence titration. Recombinant Bcl-x_L and Bcl-x_L Δ45–84 was prepared as described previously (Muchmore *et al*., 1996). The Bak peptide was purchased from PeptidoGenic Research & Co. and purified by reverse phase HPLC on a C8 column. For the titration, the fluorescence emission of the tryptophan residues of Bcl-x_L was measured with increasing peptide concentration. The fluorescence was measured on a Shimadzu RF5000U spectrofluorometer with excitation and emission wavelengths of 290 and 340 nm respectively. The fluorescence intensity was calculated with the equation $\Delta I_{obs} = X_b \times \Delta I_{(b-f)}$, where ΔI_{obs} is the difference between the observed intensity and the fluorescence intensity of the free protein, X_b is the mole fraction of the bound state and $\Delta I_{(b-f)}$ is the fluorescence difference between the bound and free forms of the protein. Mole fractions were calculated from the dissociation constant K_D, using the known initial concentrations of the protein and peptide. A least squares analysis was then performed by systematic variation of K_D and $\Delta I_{(b-f)}$. Both measurements were done using three protein concentrations and the resulting dissociation constants were averaged.

^{32}P Metabolic labeling and immunoprecipitations

Cells were labeled with 0.5 mCi [^{32}P]orthophosphate for 6 h in the presence of 1 μM okadaic acid or 10 μl ethanol vehicle control. Cells were then lysed as described previously in RIPA buffer with protease inhibitors plus 1 mM sodium vanadate, 10 mM sodium fluoride and 10 mM sodium pyrophosphate. Immunoprecipitations were performed as described above. Samples were subjected to 15% SDS–PAGE and transferred to nitrocellulose. Western blot analysis of immunoprecipitated protein was performed as described above. After allowing the ECL signal to decay, blots were air dried and placed on film to detect ^{32}P labeling.

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